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Rat Brain *N*-Acetylated α-Linked Acidic Dipeptidase Activity

PURIFICATION AND IMMUNOLOGIC CHARACTERIZATION*

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N-Acetylated α-linked acidic dipeptidase (NAALA dipeptidase) is a membrane-bound metallopeptidase that cleaves glutamate from the endogenous neuropeptide *N*-acetyl-L-aspartyl-L-glutamate. In this report, we have solubilized NAALA dipeptidase activity from synaptosomal membranes with Triton X-100 and purified it to apparent homogeneity by sequential column chromatography on DEAE-Sepharose, CM-Sepharose, and lentil lectin-Sepharose. This procedure resulted in a 720-fold purification with 1.6% yield. The purified enzyme migrated as a single silver-stained band on a sodium dodecyl sulfate gel with an apparent molecular weight of 94 kDa. Using an enzymatic stain to visualize NAALA dipeptidase activity within a gel matrix, we have confirmed that the 94-kDa band is, indeed, NAALA dipeptidase. The purified enzyme was characterized and found to be pharmacologically similar to NAALA dipeptidase activity described previously in synaptosomal membrane extracts. Using the purified NAALA dipeptidase as antigen, we have raised specific and high titer polyclonal antibodies in guinea pig. Immunocytochemical studies show intense NAALA dipeptidase immunoreactivity in the cerebellar and renal cortices.

Electrophysiologic, lesion, and immunocytochemical studies suggest that the endogenous neuropeptide, *N*-acetyl-L-aspartyl-L-glutamate (NAAG),[1] may act as a neurotransmitter/neuromodulator in the central nervous system (Blakely and Coyle, 1989). Recently a quisqualate (Quis)-sensitive peptidase activity was identified in brain membranes which cleaves NAAG to *N*-acetyl-L-aspartate (NAA) and glutamate. In a manner analogous to the synaptic inactivation of acetylcholine (Cooper *et al.*, 1986), it is hypothesized that this peptidase inactivates NAAG, and that the liberated glutamate is subsequently transported into synaptosomes by the previously characterized sodium-dependent high-affinity glutamate uptake site (Blakely *et al.*, 1986; Robinson *et al.*, 1987). Alternatively, NAAG may function as a precursor to glutamate, shifting the primary role of this peptidase to regulating glutamate availability.

This peptidase has been characterized in rat synaptosomal membranes (Robinson *et al.*, 1987; Blakely *et al.*, 1988). In this crude membrane preparation, the peptidase demonstrates remarkably high apparent affinity for its putative substrate NAAG, with a K_m = 540 nM. The enzyme is membrane-bound, stimulated by chloride ions, and inhibited by divalent metal chelators, suggesting that it is a metallopeptidase. It is enriched in synaptic plasma membranes and is primarily localized to neural tissue and kidney. Comparison of its properties to those of other known endopeptidases, aminopeptidases, dipeptidases, and acyl amino acid-releasing enzymes suggests that it is a novel peptidase (Robinson *et al.*, 1987; Blakely *et al.*, 1988). Since it is possible that NAAG is not the sole substrate for this activity *in vivo*, this peptidase was named *N*-acetylated α-linked acidic dipeptidase (NAALA dipeptidase) for its structural specificity for *N*-acetylated α-linked acidic dipeptides. Recently, it has been demonstrated that [^{3}H]NAAG is degraded by a pharmacologically similar enzyme *in vivo* (Stauch *et al.*, 1989). These data are consistent with a role for NAALA dipeptidase in the disposition of endogenous NAAG.

In this manuscript, we describe for the first time the solubilization of NAALA dipeptidase from rat membranes, its purification to apparent homogeneity, the characterization of the purified protein, the determination of its molecular weight, the production and characterization of anti-NAALA dipeptidase antibodies, and the localization of NAALA dipeptidase immunoreactivity in brain and kidney.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Chromatographic Purification of NAALA Dipeptidase—Results of the purification of rat brain NAALA dipeptidase activity are summarized in Table I. The overall purification was 720-fold with 1.6% recovery, yielding 2 mg of highly purified NAALA dipeptidase from 500 whole rat brains. Details of the solubilization and chromatographic steps are found in the miniprint supplement.

Analysis of Enzyme Homogeneity—Fig. 2 shows SDS polyacrylamide gel electrophoresis of pooled fractions at various stages in the purification. After the lentil Lectin step, there was one major silver-stained protein band migrating at 94

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[1] The abbreviations used are: NAAG, *N*-acetyl-L-aspartyl-L-glutamate; NAALA dipeptidase, *N*-acetylated α-linked acidic dipeptidase; Quis, quisqualic acid; NAA, *N*-acetyl-L-aspartate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; ELISA, enzyme linked immunosorbent assay.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Fig. 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE I

Summary of the purification of NAALA dipeptidase

NAALA dipeptidase was purified from 500 whole rat brains as described under "Experimental Procedures."

Step	Protein	Specific activity	Recovery	Purification
	mg	*pmol/mg/min*	%	*-fold*
Crude homogenate	87,000	5	100	1
Lysed synaptosomal membranes	9,000	15	31	3
Solubilized protein	2,000	71	33	14
Pooled DEAE fractions	450	160	17	32
Pooled CM fractions	20	900	4.0	180
Pooled lentil lectin fractions	2	3,600	1.6	720

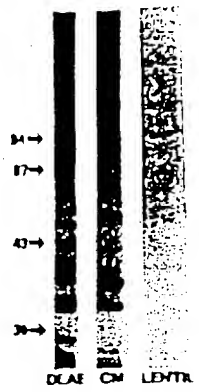

FIG. 2. SDS-polyacrylamide gel electrophoretic analysis of pooled fractions from steps during the purification of NAALA dipeptidase. *Lane A*, pooled DEAE peak I protein (10 μg of protein); *lane B*, pooled CM protein (5 μg of protein); *lane C*, pooled lentil lectin protein (0.5 μg of protein). Electrophoresis was run on a 12% gel at a constant current of 40 mA. Proteins were developed with silver staining (Wray *et al.*, 1981).

kDa, and a minor (diffuse) band migrating between 54 and 66 kDa. The literature suggests that minor contaminating protein(s) are either mercaptoethanol artifacts (Guevara *et al.*, 1982; Tasheva and Dessev, 1983) or skin keratins (Ochs, 1983).

The staining intensity of this 94-kDa band was correlated with the amount of NAALA dipeptidase activity applied to the gel. Gel electrophoresis of fractions surrounding a NAALA dipeptidase peak of activity from DEAE-Sepharose, CM-Sepharose, and size exclusion columns ($n = 4$) demonstrated that, in all cases, the 94-kDa protein band was the only band observed whose staining density coincided with NAALA dipeptidase activity (data not shown).

Although these data provide compelling evidence that the 94-kDa band is NAALA dipeptidase, it is still possible that NAALA dipeptidase is not represented by any band on the gel. Therefore, a specific enzymatic activity stain was devised to visualize NAALA dipeptidase activity within a polyacrylamide gel (Supiura *et al.*, 1977; see "Experimental Procedures"). Since a protein separated by an SDS gel (*e.g.* denatured enzyme) is highly unlikely to exhibit enzymatic activity, partially purified NAALA dipeptidase was electrophoretically separated on a nondenaturing gel and then stained for activity. Only one band was identified having NAALA dipeptidase activity (Fig. 3*A*). Since it is not possible to accurately determine molecular weight on a nondenaturing gel, this active band was excised, homogenized, and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 3*B*, *left lane*). Although

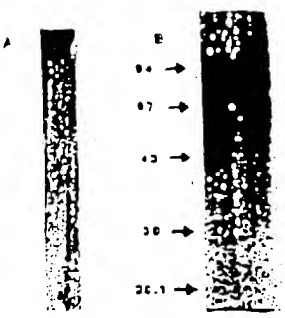

FIG. 3. Visualization of NAALA dipeptidase activity in an acrylamide gel. *A*, nondenatured gel of a semi-purified preperation stained for NAALA dipeptidase activity as described under "Experimental Methods." The resulting active band was then excised, homogenized, boiled in SDS/mercaptoethanol for 30 min, and applied to an SDS gel. *B*, the left lane is a silver-stained SDS gel of the excised NAALA dipeptidase activity band from *A*. The *right lane* is a silver-stained SDS gel of the staining components used to visualize NAALA dipeptidase activity (*e.g.* glutamate dehydrogenase, iodonitrotetrazolium violet, phenazine methosulfate). Note that the NAALA dipeptidase activity band contains only one band, migrating at 94 kDa, which cannot be attributed to the NAALA dipeptidase activity staining components.

TABLE II

Potency of compounds for inhibition/stimulation of purified NAALA dipeptidase activity

NAALA dipeptidase activity was measured as described under "Experimental Procedures" with or without inhibitors. When EGTA was tested, no exogenous cobalt was added to the assay. Peptide inhibitors were used at their previously reported IC_{50} concentrations in lysed synaptosomal membranes (Robinson *et al.*, 1987). Data are expressed as the percent of activity determined in the absence of inhibitor (control). Results are the mean of at least two experiments performed in duplicate. Except where noted, experiments were performed with L-isomers and α-linked peptides.

Compound	Concentration	Control activity
	μM	%
Inhibitors (μM)		
Asp-Glu	0.30	50
Quisqualic acid	0.48	51
Glu-Glu	0.75	51
Gly-Glu	6.0	67
γ-Glu-Glu	9.5	52
Glu	31	23
Glu-Glu-Glu	62	65
N-Acetyl-Asp	100	95
Sodium phosphate	100	57
EGTA	1000	4

five bands were revealed with this procedure, all bands, except for a 94-kDa protein, were attributed to the staining components used to visualize NAALA dipeptidase activity in the nondenaturing gel (*e.g.* glutamate dehydrogenase, iodonitrotetrazolium violet, phenazine methosulfate; Fig. 3*B*, *right lane*). These data suggest that the 94-kDa band is NAALA dipeptidase.

Properties of the Purified NAALA Dipeptidase—As was observed for activity characterized in lysed synaptosomal membranes (Robinson *et al.*, 1987), purified NAALA dipeptidase was potently inhibited by quisqualate with 50% inhibition at 0.48 μM (Table II). Peptidase activity was also inhibited by phosphate and EGTA; cobalt strongly stimulated activity. Purified NAALA dipeptidase showed a high apparent affinity for NAAG hydrolysis with a K_m of 140 nM (mean of two determinations within 10%).

Structure-activity relationships of purified NAALA dipeptidase were examined using peptide analogs of NAAG and

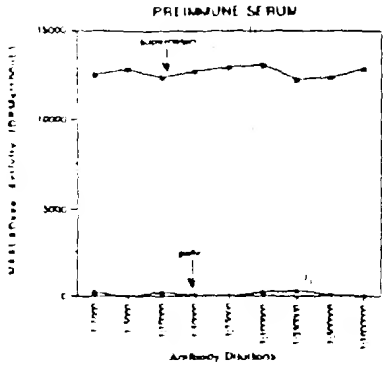

FIG. 4. *Immunoprecipitation of NAALA dipeptidase activity. Details* pertaining to the immunoprecipitation are described under "Experimental Methods." The graphs presented are one representative set of data obtained from three seperate experiments. In brief, varying amounts of preimmune and immune sera were added to crude brain membranes and incubated overnight on a rotary shaker at 4 °C. The following day, GammaBind G-Agarose was added, incubated for 3 h at 4 °C, and centrifuged. NAALA dipeptidase activity was measured in the supernatant (□) and pellet (●).

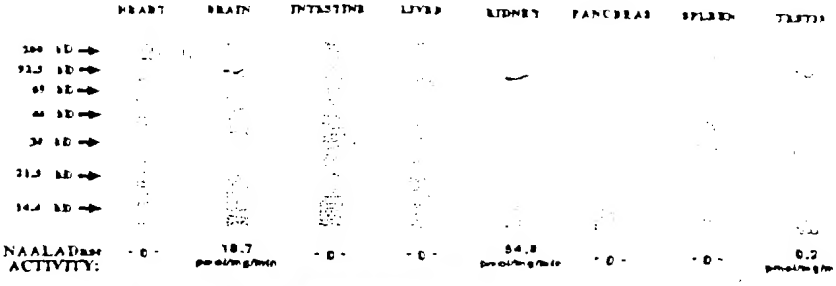

FIG. 5. Survey of the tissue distribution of NAALA dipeptidase immunoreactivity. Tissue extracts (250 μg of protein) were subject to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with immune serum against NAALA dipeptidase, followed by color development as described under "Experimental Methods." All samples shown were run in parallel. The level of NAALA dipeptidase activity identified in each region is provided.

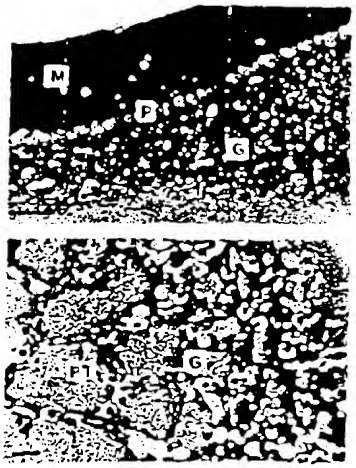

FIG. 6. Immunocytochemical localization of NAALA dipeptidase. Staining for NAALA dipeptidase immunoreactivity in rat cerebellar cortex (*top*) and rat kidney cortex (*bottom*). Note the positive staining in the molecular and granular cell layers of the cerebellar cortex and the proximal tubules and glomeruli of the kidney cortex. Details of the immunocytochemistry are described under "Experimental Procedures." Cerebellar cortex: *M*, molecular layer, *P*, Purkinje cell layer; *G*, granular layer; *Bar* = 80 μm. Kidney cortex: *PT*, proximal tubules; *G*, glomeruli; *Bar* = 300 μm.

compared with what was previously determined using a lysed synaptosomal membrane preparation (Robinson *et al.*, 1987). All peptides examined were used at their previously reported IC_{50} concentrations. The results are summarized in Table II. All data were similar to what was reported initially, except inhibition by aspartylglutamate (Asp-Glu), which was 8-fold more potent as an inhibitor of the purified enzyme than had been reported using lysed membranes (50% inhibition at 0.5 μM *versus* 2.4 μM).

Antibody Characterization—Purified NAALA dipeptidase was used to raise polyclonal antibodies in guinea pigs (see "Experimental Procedures"). Enzyme-linked immunosorbent assay, immunoprecipitation assay, and Western blotting demonstrated that the anti-NAALA dipeptidase antiserum was specific and of high titer. The titer, determined by enzyme-linked immunosorbent assay (see "Experimental Procedures"), was approximately 1:10,000 (data not shown).

Immunoprecipitation—The immune serum precipitated NAALA dipeptidase activity from solubilized brain membranes; preimmune serum did not precipitate activity (Fig. 4). Immune serum partially inhibited NAALA dipeptidase activity; therefore, at antibody dilutions less than 1:100,000 total activity using the immune serum was less than activity in the preimmune control.

Western Blotting—An SDS gel loaded with crude homogenates from rat heart, brain, intestine, liver, kidney, pancreas, spleen, and testis was transferred to nitrocellulose and probed with anti-NAALA dipeptidase immune serum as described under "Experimental Procedures." The immune serum recognized a single 94-kDa band in brain, kidney, and testis only, consistent with the localization of NAALA dipeptidase activity (Fig. 5). No staining was detected using preimmune serum.

Immunocytochemistry—Immunocytochemical experiments revealed NAALA dipeptidase-positive staining in the molecular and granule cell layers of the cerebellar cortex; the Purkinje cell layer was devoid of immunoreactivity (Fig. 6, *top*). In the rat kidney, NAALA dipeptidase-positive staining was detected in the proximal tubules and glomeruli of the renal cortex; the distal tubules were essentially devoid of immunoreactivity (Fig. 6, *bottom*). No immunostaining was revealed using preimmune serum, even at 10-fold higher concentrations. In addition, preadsorption of the antiserum with purified NAALA dipeptidase (0.05 mg/ml) completely abolished immunochemical staining.

DISCUSSION

Brain NAALA dipeptidase activity was solubilized with Triton X-100 and sequentially purified with ion-exchange and

lentil lectin affinity chromatography (Fig. 1). DEAE-Sepharose resolved NAALA dipeptidase activity into two peaks; however, both peaks were pharmacologically and kinetically similar. Since DEAE peak 1 was the predominant species (>65% of eluted activity), it was employed for further purification. NAALA dipeptidase activity (peak 1) did not interact with DEAE-Sepharose at pH 7.9 and bound to CM-Sepharose at this same pH, suggesting that this protein has an unusually high isoelectric point (pI); most proteins have pI values below pH 7.4 (Righetti and Caravaggio, 1976). Chromatofocusing chromatography confirmed this finding, revealing a pI for NAALA dipeptidase of approximately pH 9.0. Following lentil lectin chromatography, the purified preparation showed one major silver-stained band on SDS-polyacrylamide gel electrophoresis migrating at 94 kDa and a minor (diffuse) band migrating between 54 and 66 kDa. The literature suggests that this minor broad band is either a mercaptoethanol artifact (Guevara *et al.*, 1982; Tasheva and Dessev, 1983) or skin keratins (Ochs, 1983). In fact, we have seen this diffuse band in lanes run with sample buffer alone.

To demonstrate that the 94-kDa protein represented NAALA dipeptidase, activity applied to an SDS gel was correlated with protein staining intensity of this band. In all gels, the 94-kDa band was the only band observed whose staining density coincided with the amount of applied NAALA dipeptidase activity. Furthermore, NAALA dipeptidase activity was visualized directly in a nondenaturing gel (Fig. 3*A*). NAALA dipeptidase activity demonstrated a low degree of migration into the nondenaturing gel, consistent with its high isoelectric point. This resulting activity band was excised from the nondenaturing gel and run on an SDS gel. Protein staining again revealed a single unique band at 94 kDa (Fig. 3*B*). Together, these data strongly suggest that this 94-kDa band is NAALA dipeptidase.

Size exclusion chromatography of the purified and semipurified protein show that NAALA dipeptidase migrates consistent with a molecular mass of 225 kDa, although larger species were occasionally observed. Both protein and activity gels demonstrate that peptidase has a denatured molecular mass of approximately 94 kDa. Therefore, it is possible the NAALA dipeptidase is a dimer composed of two identical subunits; alternatively, the larger species identified with size exclusion chromatography may represent protein-detergent complexes.

Properties of the purified protein were similar to activity previously characterized in lysed synaptosomal membranes (Robinson *et al.*, 1987), demonstrating that these properties are due to direct interaction with NAALA dipeptidase and are not indirectly mediated by other proteins present in the membrane preparation. The potent inhibition of peptidase activity by quisqualate suggests that some of its actions, which were previously attributed to interaction with a subclass of glutamate receptors (Robinson and Coyle, 1988), may be due to inhibition of NAALA dipeptidase. EGTA sensitivity and cobalt stimulation support initial data suggesting that NAALA dipeptidase was a metallopeptidase. Similar to activity in lysed synaptosomal membranes, purified NAALA dipeptidase displayed structure specificity for *N*-acetylated α-linked acidic dipeptides (Table II). Finally, the purified NAALA dipeptidase displayed a remarkably high apparent affinity for its putative substrate, NAAG, with a K_m of 140 nM.

The availability of purified protein permitted the production of anti-NAALA dipeptidase antisera. The results presented in this study demonstrate that the polyclonal antibodies raised in guinea pig are remarkably selective, of high titer, and capable of recognizing both native and denatured NAALA dipeptidase. Western analysis of gels loaded with crude brain homogenates revealed that the antisera exclusively recognized the 94-kDa band. Besides brain NAALA dipeptidase, the antibodies cross-reacted with kidney and testis NAALA dipeptidase; no immunoreactivity was observed in regions devoid of NAALA dipeptidase activity (Fig. 5). The antisera inhibited NAALA dipeptidase activity, although not completely (70% inhibition at 1:100 dilution), and was capable of precipitating NAALA dipeptidase activity from a crude brain extract (Fig. 4).

Using this selective and specific antisera, NAALA dipeptidase immunoreactivity was localized to the glomeruli and proximal tubules of the kidney cortex (Fig. 5*B*). This localization is consistent with micropunch analysis of NAALA dipeptidase activity,[2] which found that the vast majority of NAALA dipeptidase activity was localized to the renal cortex (kidney cortex = 166 pmol/mg/min *versus* kidney medulla = 10 pmol/mg/min). Interestingly, other brain peptidases, angiotensin converting enzyme and enkephalinase (Schulz, 1988; Teuc, 1988) have also been localized in the glomeruli and proximal tubules of the kidney, areas where their putative brain substrates are not found. In the neural tissue, NAALA dipeptidase immunoreactivity was found in areas reported previously to contain NAAG (Blakely and Coyle, 1989).

NAALA dipeptidase is a novel enzymatic activity involved in NAAG hydrolysis. In this study, we have solubilized and purified rat brain NAALA dipeptidase to apparent homogeneity, developed specific anti-NAALA dipeptidase antiserum, and have begun to map its distribution in rat brain and kidney. We anticipate using the antiserum to fully determine its renal and neuronal localization and to screen a cDNA library to obtain the NAALA dipeptidase clone.

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[2] S. S. Richards, G. Forloni, M. B. Robinson, and J. T. Coyle, unpublished observation.

RAT BRAIN N-ACETYLATED ALPHA-LINKED ACIDIC DIPEPTIDASE ACTIVITY: PURIFICATION AND IMMUNOLOGIC CHARACTERIZATION

EXPERIMENTAL PROCEDURES

RESULTS

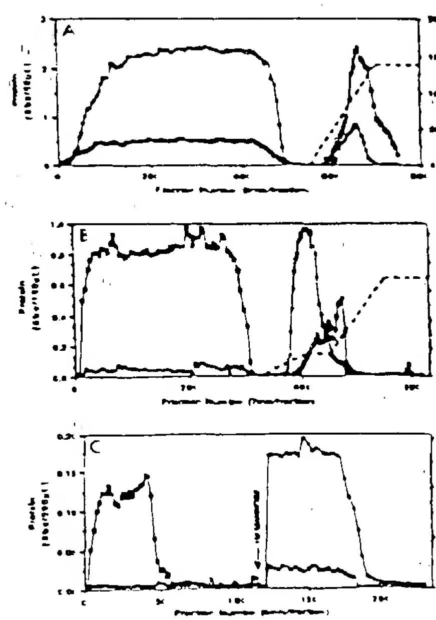

Fig. 1. Chromatographic purification of NAALA dipeptidase activity.